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(21) International Application Number: PCT/US95/07933 (22) International Filing Date: 21 June 1995 (21.06.95) (30) Priority Data: 08/322,624 13 October 1994 (13.10.94) US (71) Applicant: WISCONSIN ALUMNI RESEARCH FOUNDATION [US/US]; P.O. Box 7365, Madison, WI 53707-7365 (US). (72) Inventors: JOHNSON, Eric, A.; 3901 Council Crest, Madison, WI 53711 (US). GOODNOUGH, Michael, C.; 6914 Harvest Hill Road, Madison, WI 53717 (US). (74) Agents: HAAS, George, E. et al.; Quarles & Brady, 411 East Wisconsin Avenue, Milwaukee, WI 53202 (US).		(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG). Published <i>With international search report.</i>
(54) Title: PHARMACEUTICAL COMPOSITION OF BOTULINUM NEUROTOXIN AND METHOD OF PREPARATION (57) Abstract Pharmaceutical compositions of botulinum neurotoxin containing higher specific toxicity and increased stability at higher temperatures than currently available preparations.		

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PHARMACEUTICAL COMPOSITION OF BOTULINUM NEUROTOXIN
AND METHOD OF PREPARATION

Field of the Invention

The present invention relates to botulinum toxin. More
5 particularly, it relates to novel pharmaceutical compositions
containing botulinum toxin and a method for preparing the
compositions.

Background of the Invention

The most serious form of bacterial food poisoning is
10 botulism which is caused by neurotoxins produced by
Clostridium botulinum. The toxins are usually preformed by
the causative organism in foods and subsequently absorbed
through the intestinal tract and transported via the
circulatory system to motor nerve synapses where their action
15 blocks normal neural transmissions. Various serotypes of *C.*
botulinum produce neurotoxins with similar toxic activity but
which differ antigenically. Serotype A toxin is the
predominant cause of botulism in the United States while type
B toxin is the most prevalent in Europe.

20 Crystalline type A botulinum toxin complex was prepared
in 1979 by E. J. Schantz of the Food Research Institute/
Department of Food Microbiology and Toxicology at the
University of Wisconsin-Madison. It has been used medically
to treat hyperactive muscle disorders such as strabismus,
25 blepharospasm, and spasmodic torticollis. Treatment involves
injection of nanogram quantities of the toxin directly into
the hyperactive muscles. The toxin inhibits the release of

acetylcholine across the synaptic junction causing a decrease in the activity of the injected muscles.

Type A neurotoxin produced by *C. botulinum* is present as part of a complex of at least seven different noncovalently bound proteins. High quality type A toxin complex has a specific toxicity of 3×10^7 mouse intraperitoneal 50% lethal doses (LD_{50}) per mg. The purified neurotoxin, that is the neurotoxin that has been chromatographically separated from the other proteins of the toxin complex, has a specific toxicity of 9×10^7 to 1×10^8 LD_{50} per mg. In the medical field, a unit (U) is considered to be 1 LD_{50} . Toxin titers are determined in female, white mice, 18-22g in weight according to the method of Schantz and Kautter as described in Association of Official and Analytical Chemistry, vol. 61, p. 96, (1978).

A major drawback to using botulinum toxin in treatment of hyperactive muscle disorders is development of antibodies or other types of immunity by patients. The toxin is recognized by patient's immune systems as foreign and stimulates antibody production. This renders treatment of the various hyperactive muscle disorders with botulinum toxin ineffective. One way to reduce the number of patients developing neutralizing antibodies would be to develop a more shelf-stable product with a higher specific activity following lyophilization. Such a formulation would result in a product that is not as antigenic as the currently available product and lesser quantities of toxin would be required for treatment.

Botulinal toxin is very susceptible to denaturation due to surface denaturation, heat, and alkaline conditions. Lyophilization or freeze-drying of botulinal toxin is the most economically sound and practical method of distributing the product in a form that is stable and readily used by the clinician. The current commercial type A botulinal toxin product is made by combining up to 500 ng/ml of type A toxin complex in 5.0 mg/ml human serum albumin (HSA) with 9.0 mg/ml sodium chloride at a pH of 7.3. After dissolution, 0.1 ml is dried to obtain 100 ± 30 active U of toxin, 0.5 mg of HSA, and 0.9 mg of sodium chloride per vial. This product has a saline concentration of 0.9% when reconstituted in 1.0 ml of dH_2O . The current commercial formulation which employs the toxin complex has a specific toxicity of about 2.5 U/ng after drying. The considerable loss (up to 90%) of activity during drying causes the formation of inactive toxin that serves as a toxoid inciting antibody formation.

A rabbit model in which repetitive injections of various type A toxin preparations have been given to simulate the treatment of a focal dystonia has been used to assess the immunogenicity of various toxin preparations. The model consists of injecting albino rabbits with sub-lethal doses of the toxin over a period of time and assaying the serum of the animals for the ability to neutralize a small but carefully quantitated amount of purified type A toxin. Our results show that the product presently available in the United States which has the lowest specific toxicity of all preparations tested is the most antigenic of all the preparations tested to

date. These results indicate that high specific activity preparations reduce the probability of patients developing neutralizing antibodies. It obviously would be desirable to have higher specific activity preparations than those
5 currently available.

The current commercial product must be stored at a temperature of -10°C or less to maintain the labelled potency for the one year shelf life. It also would be advantageous to have a product that could be stored at higher temperatures
10 (i.e. room temperature). This would facilitate more practical shipping and storage of the toxin.

Brief Summary of the Invention

We have discovered that pharmaceutical compositions made from a liquid formulation containing essentially pure
15 botulinum type A neurotoxin, human serum albumin (HSA), and trehalose provides for the improved recovery of active toxin following lyophilization ($>80\%$). The use of the pure neurotoxin instead of the toxin complex, which is used commercially, reduces the amount of toxin required to obtain
20 the necessary number of active U per vial as mandated by the U.S. Food and Drug Administration. This improvement also reduces the amount of inactive toxin (toxoid) in each vial and thereby lessens the possibility of antibody formation after injection of the preparation into patients.

25 We also have discovered that the compositions obtained by adding trehalose to the pre-lyophilization formula increases the glass transition temperature of the dried material and

thereby increases the usable storage temperature. The addition of the trehalose surprisingly enhances temperature stability of the dried toxin and lessens both the risk of loss in potency with corresponding degradation during storage and shipment with a consequent increase in antigenic potential due to temperature abuse. In addition to trehalose, we have discovered that the polyhydroxy compound maltotriose and possibly other polyols can be used.

Description of Preferred Embodiment

10 The preferred pharmaceutical compositions of the present invention have the following composition:

Botulinum Type A Neurotoxin (>95% purity)

Trehalose, 10 mg/vial

Serum albumin, 0.5 mg/vial

15 Water for Injection, 0.1 - 0.5 ml

In addition to human serum albumin, other known stabilizing proteins including bovine serum albumin, can be used in the compositions of the present invention.

The Hall A strain of type A *C. botulinum* (deposited with the ATCC) is used to produce type A toxin. This strain is routinely used for production of type A botulinum toxin due to high toxin titers and the rapid onset of cell lysis (usually within 48 h).

For toxin production, cultures of the Hall A strain are grown statically in 10-20 liter volumes of toxin production medium (TPM) consisting of 2.0% NZ amine or TT (Sheffield

Laboratories, Norwich, NY), 1.0% yeast extract (Difco), and 0.5% dextrose, pH 7.37.4, for 5-7 days at 37°C.

To prepare essentially pure type A neurotoxin, the type A toxin complex is first purified according to the method
5 described in the Ph.D. thesis of M. C. Goodnough (Goodnough, M.C. 1994, Characterization and stabilization of *Clostridium botulinum* toxin for medical use. Ph.D. thesis, UW-Madison, as adapted from Tse et al. 1982)

Type A neurotoxin is purified from the associated non-
10 toxic proteins of the complex by a modification of the method of Tse et al. (1982) (Goodnough, M.C., 1994, Thesis, UW, Wisconsin). Toxin complex is recovered from the DEAE-Sephadex A50 (Sigma Chemical Co., St. Louis, MO), pH 5.5, column and is precipitated by addition of 39 g of solid ammonium
15 sulfate/100ml. The precipitated toxin complex is collected by centrifugation, dialyzed against 25 mM sodium phosphate, pH 7.9, and applied to a DEAE-Sephadex A50 column equilibrated with the same buffer. Toxin is separated from the non-toxic proteins of the complex and eluted from the column with a
20 linear 0-0.5M sodium chloride gradient. Partially purified neurotoxin is recovered from the DEAE-Sephadex A50 column at pH 7.9 and dialyzed against 25 mM sodium phosphate, pH 7.0. The dialyzed toxin is applied to SP-Sephadex C50 (Sigma Chemical Co.) in 25 mM sodium phosphate, pH 7.0.
25 Contaminating material does not bind to the column under these conditions. The neurotoxin is eluted with a linear 0-0.25 M sodium chloride gradient. The neurotoxin can be further

purified by metal affinity chromatography, gel filtration or other methods of protein chromatography.

For lyophilization, toxin samples are diluted in the excipients (stabilizing compounds) to be tested (Sigma
5 Chemical Co.), 0.1 ml or 0.5 ml aliquoted into 2 ml glass vials (Fisher Scientific Co., Pittsburgh, PA), the Teflon lined screw cap closures fastened loosely, and the samples are quickly frozen in liquid nitrogen. The frozen samples are placed into a lyophilization flask which is then immersed in
10 liquid nitrogen. The flask is then connected to a laboratory freeze-drier (Virtis Freezmobile 12, Virtis Co., Inc., Gardiner, NY). When the pressure drops below ca. 60 mTorr, the liquid nitrogen jacket is removed. Pressure is maintained at or below 30-60 mTorr and condenser temperature constant at
15 -60°C. Samples are allowed to come to room temperature and drying continued at ambient temperature over the next 18-24 h. At that time the flask is removed and the vials tightly capped. Vials were assayed for toxicity and recovery calculated within 1-3 days (adapted from Goodnough and
20 Johnson, 1992).

Vials of lyophilized type A neurotoxin and type A toxin complex were stored at various temperatures to investigate the effect of added excipients on the shelf-stability of the dried material. In these cases, the tightly capped vials were
25 placed into plastic bags, sealed and stored at various temperatures (-20, 4, or 37°C) and the contents assayed for toxicity at various time points. The lyophilized preparations were usually reconstituted in 1.0 ml of distilled water. The

use of 0.85% saline for reconstitution gave equivalent results. The white cake dissolved immediately and was mixed by gentle inversion of the vials. The resulting solution was transparent and contained no particulates. This solution was
5 titrated by the same method used for the prelyophilization solution. The percent recovery (calculated as number of mouse intraperitoneal lethal doses per vial after lyophilization divided by the number of mouse intraperitoneal lethal doses before lyophilization X 100) represent averages of trials done
10 in at least duplicate. The variation in independent assays was ca. $\pm 20\%$.

The percent recoveries of active toxin determined within 2-3 days following lyophilization of type A toxin complex and purified type A neurotoxin in various excipient combinations
15 are shown in Tables 1 and 2.

The primary advantages of preferred compositions of the present invention are their high percentage recovery of biologically active neurotoxin and their long-term stability (shelf life) at temperatures above 0°C. In contrast, the
20 current commercial product has a low percentage recovery of biologically active neurotoxin and must be stored at temperatures of -10°C or less.

Table 1. Effect of excipients on recovery of toxicity of *Clostridium botulinum* type A toxin complex after lyophilization of 0.1 ml of each solution.

<u>Excipients</u>	<u>Starting Toxin concentration^a</u>	<u>pH</u>	<u>%recovery^b</u>
sodium phosphate ^c	50, 100, 1,000	5.0, 6.0, 6.8	<10
bovine serum albumin/sodium chloride ^d	100	6.4	10
bovine serum albumin ^e	100, 1,000	6.4	88, 75
bovine serum albumin/citrate ^f	100, 1,000	5.0	>90, >90
bovine serum albumin/phosphate ^g	100, 1,000	5.5	>90, >90
bovine serum albumin/phosphate ^g	1,000	7.3	60
bovine serum albumin/phosphate ^h	1,000	6.0	>90
human serum albumin ⁱ	100, 1,000	6.4-6.8	>90, >90
alpha-lactalbumin ^j	1,800	6.1	>78
lysozyme ^j	1,800	5.3	>78
gelatin ^j	1,800	6.3	>78
bovine serum albumin/trehalose ^k	500	5.7	>90
bovine serum albumin/sucrose ^l	325	6.6	65
bovine serum albumin/maltotriose ^m	250	7.0	>80

^a Type A mouse lethal doses/vial before lyophilization;

^b %recovery = (number mouse lethal doses after lyophilization/number mouse lethal doses prior to lyophilization) x 100;

^c 50mM sodium phosphate;

^d bovine serum albumin (5.0mg/ml), sodium chloride (9.0mg/ml);

- ^e bovine serum albumin (9.0mg/ml);
- ^f bovine serum albumin (9.0mg/ml), 50mM sodium citrate;
- ^g bovine serum albumin (9.0mg/ml), 50mM sodium phosphate;
- ^h bovine serum albumin (9.0mg/ml), 50mM potassium phosphate;
- ⁱ human serum albumin (9.0mg/ml);
- ^j concentration = 9.0mg/ml;
- ^k 9.0mg/ml bovine serum albumin, 100mg/ml trehalose;
- ^l 9.0mg/ml bovine serum albumin, 250mg/ml sucrose;
- ^m 9.0mg/ml bovine serum albumin, 100mg/ml maltotriose. (adapted from Goodnough and Johnson, 1992).

Table 2. Recovery of activity following lyophilization of purified *Clostridium botulinum* type A neurotoxin.

<u>Excipient combination</u>	<u>Starting Toxin concentration^b</u>	<u>pH</u>	<u>%recovery^c</u>
bovine serum albumin	200	6.4	90
human serum albumin	1,000	6.4	90
bovine serum albumin, trehalose	500	5.7	>90
bovine serum albumin, sucrose	325	6.6	50
bovine serum albumin, maltotriose	250	7.0	>80

^a bovine and human serum albumin concentration was 9.0 mg/ml, carbohydrate concentration was 100 mg/ml in all cases except sucrose which was 250 mg/ml;

^b mouse intraperitoneal lethal doses/vial;

^c (number of mouse lethal doses/vial after lyophilization, number of mouse lethal doses before lyophilization) x 100.

It was found that formulations used for lyophilization had a marked effect on the recovery of toxin. The most critical factor was the absence of sodium chloride in the solution in combination with a pH less than 7.0. In the best cases, recovery of active toxin was >90% following lyophilization.

The addition of trehalose in particular, allowed the recovery of active type A neurotoxin following lyophilization and storage at temperatures in excess of those required for storage of the commercially available type A toxin complex (37°C versus -10°C).

The antigenicity of various toxin preparations (containing low or high specific toxicities) was evaluated in rabbits by repetitive injection of sublethal doses of toxin simulating treatment of a focal dystonia with botulinal toxin. The samples were standardized to contain the same number of active lethal doses in order that the immune response from the rabbits could be compared. The samples with the highest specific activity were those consisting of purified type A neurotoxin (96 U/ng) while the lowest were the commercially available Botox® samples (4.3 U/ng).

Total toxin concentration for each preparation (i.e. both active and inactive) was determined using an enzyme-linked immunosorbent assay (ELISA) specific for type A botulinal toxin. The ELISA assays performed on Botox® and ASB indicated that Botox® had an average specific toxicity of 4.3 U/ng and ASB had an average specific toxicity of 17.3 U/ng after

reconstitution. Type A toxin complex used in these assays had a specific activity of 18 U/ng.

Serum samples were drawn on the days shown in Table 3 and titrated for the ability to neutralize 5.6 U of essentially
5 pure type A neurotoxin. Total toxin concentration (active + inactive) in each preparation was determined by ELISA following reconstitution except in the cases of A neurotoxin and A complex which were not lyophilized.

Table 3. Immune response of rabbits to sub-lethal doses of type A botulinum toxin.

Day	A neurotoxin	A complex	Botox I*	Botox II*	ASB I*	ASB II*
0		0	0	0	0	0
29		28	21	21	21	21
42		35	35	35	35	35
56		56	49	49	49	46
69		69	60(1:1) ^a	63(1:1) ^a	60	60
88		88			(no antibodies detected)	67
107		109		77(1:2)		81
118		118		84(1:4)		95(1:1) ^a
	(No antibodies detected)	(No antibodies detected)				
Total ng of toxin:	0.92	5.12	18.3	25.41	4.075	6.52

*Two separate animal trials labeled I and II are represented.

^a All antibody samples were titrated against 5.6 mouse lethal doses of purified type A neurotoxin according to the following: 0.5ml serum + 0.1ml containing 5.6 LD₅₀ type A toxin + 0.6ml gel-phosphate, pH 6.4. The solution was incubated at room temperature for 30-60 minutes. Two mice per two fold dilution were injected intraperitoneally with 0.5ml of serum + toxin mixture. Dilutions which neutralized the toxin challenge are indicated in brackets. The last value in the numerical column indicates the final day of sampling. Numbers in parentheses indicate final dilution which neutralized toxin challenge.

These results show that the immune response of rabbits to botulinal toxin is dependent on concentration of the toxin (active + inactive) injected as well as the number of times the animal is exposed to that concentration. From these results it follows that the higher the specific activity of the lyophilized/reconstituted toxin product, the less antigenic material the patient is exposed to and the smaller the chances of patients developing neutralizing antibodies. Thus, it is advantageous to have pharmaceutical compositions of lyophilized essentially pure neurotoxin which permit the recovery of a high percentage of the starting activity and contain trehalose for storage of the dried product without degradation at temperatures above that of the currently available product are shown.

It will be apparent to those skilled in the art that a number of modifications and changes can be made without departing from the spirit and scope of the present invention. Therefore, it is intended that the invention be limited only by the claims.

We claim:

1. A pharmaceutical composition comprising:
 - (a) isolated, essentially pure type A botulinum neurotoxin having a specific toxicity of greater than 80 U/ng;
 - 5 (b) a stabilizing protein;
 - (c) an effective amount of a polyhydroxy compound which stabilizes the neurotoxin and improves the shelf life of the composition at temperatures up to about 37°C; and
 - (d) water for injection,
 - 10 said composition having a pH of less than about 7.
2. A composition of claim 1 in which the stabilizing protein is human serum albumin.
3. A composition of claim 1 in which the polyhydroxy compound is selected from trehalose, maltotriose and related
15 compounds.
4. A composition of claim 1 in which the botulinum neurotoxin has a purity of greater than about 95%.
5. A lyophilized pharmaceutical composition of type A botulinum neurotoxin which is stable for up to four months at
20 about 37°C without the neurotoxin losing potency, said composition containing essentially pure type A botulinum neurotoxin and an effective amount of trehalose to stabilize the neurotoxin.

6. A composition of claim 5 which contains greater than 80 units per nanogram of the active, essentially pure neurotoxin.

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K38/16

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JOURNAL OF NEUROLOGY, vol. 239, no. 1, January 1992 BERLIN, DE, pages 16-20, P. HAMBLETON 'CLOSTRIDIUM BOTULINUM TOXINS: A GENERAL REVIEW OF INVOLVEMENT IN DISEASE, STRUCTURE, MODE OF ACTION AND PREPARATION FOR CLINICAL USE.' see page 18, paragraph 6 - page 19, paragraph 1 ---	1-6
A	APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 58, no. 10, October 1992 WASHINGTON, D.C., US, pages 3426-3428, M.C. GOODNOUGH ET AL. 'STABILIZATION OF BOTULINUM TOXIN TYPE A DURING LYOPHILISATION.' see table 1 --- -/--	1-6

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

25 September 1995

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Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Ryckebosch, A

INTERNATIONAL SEARCH REPORT

Application No

PCT/US 95/07933

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>EP,A,0 593 176 (WISCONSIN ALUMNI RESEARCH FOUNDATION) 20 April 1994 see table 1</p> <p>-----</p>	1-6

INTERNATIONAL SEARCH REPORT

Information on patent family members

Intern:	Application No
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PCT/US 95/07933

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-593176	20-04-94	JP-A- 6192118	12-07-94
